ASSAYING IN VIVO T-CELL RESPONSE UPON DNA VACCINATION

Field of the invention

The invention relates to an assay to monitor the immune response generated following administration of nucleic acid encoding an antigen, using a mammal into which transgenic T-cells expressing T-cell-receptor specific for the antigen have been transferred.

Background to the invention

Nucleic acids and in particular DNA encoding antigens are now being proposed to replace conventional protein or attenuated organisms in vaccine formulations. Nucleic acid, preferably DNA, encoding a particular antigen can be administered by a number of different means. The peptide or protein encoded by the nucleic acid is then expressed in the body and subsequently presented to the immune system. Preferably, a protective immune response is generated. A summary of nucleic acid vaccination is provided by Robinson H. L. and Torrer C. A. T. "DNA vaccines" in Seminars in Immunology 1997, 9: 271-283.

Methods are required to monitor the immune response which is generated following DNA vaccination. It is desirable that the assessment of the immune response is not only quantitative but is also qualitative. Desirably any assessment may be used to establish the nature of the immune response, such as humoral or cellular immune responses and the type of cellular response which has been produced.

It is often difficult to monitor cellular immune responses generated in response to a specific antigen. In general, it is not possible to monitor antigen-specific T-cell responses *in vivo* following immunisation. In particular, it is difficult to determine whether signals generated derive from the antigen-specific T-cells or other cells effected by agents produced by these cells. Pape *et al* Immunological Reviews 1997 156:67-78 describe a technique to study T-cell activation *in vivo*. The technique, referred to as adoptive transfer, uses transgenic T-cells which have been transformed to express an antigen-specific T-cell receptor. These transgenic T-cells are transferred into syngeneic mice which are subsequently immunised with the same antigen. After administration of the

antigen, clonal expansion of the transferred T-cell population was monitored. This T-cell population was also extracted and cytokine production in response to restimulation with antigen was monitored *in vitro*.

Summary of the invention

It has been found that an adoptive transfer technique may be used to monitor T-cell responses *in vivo* in response to administration of a nucleic acid vaccine. The technique has proved surprisingly useful in monitoring the nature of the T-cell responses and for example may be useful in differentiating between the production of a Th1 and a Th2 response.

In accordance with the invention there is provided a method for assessing a T-cell response to nucleic acid administration comprising:

a) transferring into a mammal T-cells expressing a T-cell receptor for an antigen (TCR-T-cells);
b) administering nucleic acid encoding said antigen to the mammal; and
c) assessing an immune response associated with said TCR-T-cells.

In a preferred aspect, the TCR-T-cell population is monitored to assess clonal expansion of said population of T-cells. Alternatively or additionally, the population of T-cells may be restimulated with said antigen *in vitro*. Subsequently, cytokine production, T-cell surface markers or proliferation may be monitored. Preferably, levels of interferon-(and/or IL-5 are monitored. The T-cell response to be assessed may be assessment of a T-helper cell response, and the type of T-helper response generated, assessment of a cytotoxic T-cell response or assessment of a memory T-cell response.

In the figures

Figure 1. Clonal expansion of TCR transgenic T cells. The percentage of KJ1-2.6+ CD4+ cells of the total T cell lymphocyte population was measured on FACS from lymph node T cells taken at intervals after primary immunisation. ATE6 and ATE8 represent two separate experiments. Each point is the mean ± SEM of 3 mice per group.

Figure 2. T cell proliferation of lymph node T cells taken at intervals after primary immunisation and restimulated *in vitro* with cognate peptide. After 2-3 days *in vitro* restimulated with cognate peptide the cells were pulsed overnight with tritiated thymidine and cells harvested and radioactive counts measured. Each point is the mean ± SEM of 3 mice per group.

Figures 3-5. Cytokine concentrations in culture medium supernatant of lymph node T cells taken at intervals after primary immunisation and restimulated *in vitro* with cognate peptide. Supernatants were collected at intervals during *in vitro* culture and assayed for IL-2, IFN-γ or IL-5. Each point is the mean ± SEM of 3 mice per group.

Detailed description of the invention

The present invention provides a method for assessing T-cell responses following nucleic acid administration. The nucleic acid administration is carried out on a mammal into which a desired T-cell population has been transferred. The resulting T-cell responses can be monitored directly *in vivo* through an assessment of the expansion of the T-cell population or substances produced thereby *in vivo*. Alternatively, a sample containing a T-cell population can be removed after nucleic acid administration for further *in vitro* analysis.

The method may also be used to assess the responses generated following administration of more than one nucleic acid encoding antigen. For example, the method may be used to assess the effect of administering nucleic acid encoding 2 or more antigens. Alternatively nucleic acids such as DNA encoding an immuno modulator may be administered. Examples of such immuno modulators include cytokines, chemokines and co-stimulatory surface molecules. Alternatively, a chemical immunomodulator may be administered. Such additional nucleic acids encoding antigens or immunomodulators or other chemical modulators may be administered at the same time as administration of nucleic acid encoding antigen, before such administration, or continue throughout the course of the assay.

The present invention may be used to assess T-cell responses to assist in identifying whether a protective immune response may be generated. The assays may also be used where a tolerising or anergic response is desired.

In accordance with the method, a T-cell population is transferred into a mammal (adoptive transfer). Preferably, the mammal is a non-human mammal. More preferably, the mammal is a rodent and most preferably is a mouse.

The T-cell population transferred into the mammal express a T-cell receptor specific for an antigen. Such cells are herein after referred to as TCR-T-cells. T-cells may be CD4+ or CD8+ cells. Such cells may be produced *in vitro*, by transforming splenocytes with polynucleotide such as DNA encoding a specific T-cell receptor. Alternatively, T-cells expressing a specific T-cell receptor may be obtained from a transgenic mammal. Such T-cells may be expanded *in vitro* to provide a suitable population for administration to said mammal. Such cells could also be produced by clonal expansion of T-cells clones by restimulation of pre-immune splenocytes with antigen presenting cells presenting antigen specific for the T-cell receptor. Pape *et al*, for example, describes methods of producing a suitable population of T-cells.

TCR-T-cells may be naive (ie. not have been stimulated with cognate peptide) or antigen-experienced prior to transfer. Antigen-experienced TCR-T-cells may also be of a defined phenotype, such as Th1, Th2, Thpp, Tem, Tcm, or effector T cells prior to transfer. Phenotypically defined TCR-T-cell populations would be generated using methods described in RA Seder and WE Paul "Acquisition of lymphokine producing phenotype by CD4+ T cells" in Annual Review of Immunology 1994, 12, 635-73.According to one embodiment, before transfer, splenocytes are treated with anti-CD8 monoclonal antibodies plus complement before transfer to eliminate $CD4^-$, CD8+, KJI-26 + cells that are not responsive to the antigen and could confuse the analysis.

Typically, T-cells are administered to the animal in the range of 1×10^5 - 1×10^8 cells, more preferably $1 \times 10^6 - 5 \times 10^7$, and most preferably in the range of $1 \times 10^6 - 2 \times 10^7$ cells per mouse. Those skilled in the art would readily be able to assess a suitable T-cell population depending on the mammal being used. Preferably, the T-cell population is transferred to the mammal by intravenous, subcutaneous, intradermal and intraperitoneal injection. The T-cell population is generally administered from 1 day to 4 weeks prior to administration of nucleic acids. Preferably, adoptive transfer is carried out between 1 to 7 days prior to nucleic acid administration.

In one embodiment, the T-cell population for transfer may express two or more T-cell receptors, specific for two or more antigens. This population may comprise T-cells, each of which expresses two types of T-cell receptor each specific for an antigen. Alternatively the population may comprise T-cells some of which express one type of antigen-specific T-cell receptor, and some of which express a different type of antigen-specific T-cell receptor.

Such T-cell populations may be useful to look at the different T-cell responses to different epitopes of the same protein or to look at the interaction between the T-cell populations.

Nucleic acid encoding the antigen is administered to the mammal. Nucleic acid, such as RNA or DNA, and preferably, DNA, is provided in the form of a vector which may be expressed in the cells of the mammal.

Preferably, suitable expression elements for expression of the antigen are provided. The antigen may be a complete protein or a part of a protein containing an epitope. The antigen may be from a virus, prokaryote, or a eukaryote, for example, from a bacterium, a yeast, a fungus or a eukaryotic parasite. The antigen may be from an extracellular or intracellular protein. Antigens which may generate a tolerising or anergic response may also be used. For example the assay may be used to monitor T-cell responses occurring in asthma, rheumatoid arthritis or autoimmune diseases such as multiple sclerosis.

Nucleic acid may also be provided in the form of viruses and bacteria genetically modified to express the antigen. Examples of such viruses include adeno viruses, alpha viruses and DISC-HSV-2 virus or salmonella.

Nucleic acid encoding the antigen may be administered to the animal by any available technique. For example, the nucleic acid may be introduced by injection, preferably intradermally, subcutaneously or intramuscularly. Alternatively, the nucleic acid may be delivered directly across the skin using a nucleic acid delivery device such as particle-mediated gene delivery. This technique is also known as 'gene gun' technology and is described in US Patent No. 5371015. According to the particle-mediated gene delivery technique, particles, typically gold beads coated with a nucleic acid, are accelerated at speeds sufficient to enable

them to penetrate a surface of a recipient (e.g. skin) by means of discharge under high pressure from a projecting device. Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding. Microseeding is described in US-5,697,901.

The antigen may be administered topically to the skin, or to the mucosal surfaces for example by intranasal, oral, intravaginal, intrarectal administration.

Additional antigens or immuno modulators may be administered as nucleic acid encoding the additional antigens or immuno modulators. Such nucleic acid may be expressed as a fusion protein with the antigen to be administered. Alternatively, such additional antigens or immuno modulators may be expressed as separate proteins but incorporated within the same vector as nucleic acids encoding antigen. Alternatively, separate nucleic acid constructs may be provided for expression of the additional antigens or immunomodulators which may be administered together with the nucleic acid encoding antigen or may be administered separately. Chemical immunomodulators may also be administered. Such additional antigens or immunomodulators may be administered at the same time as nucleic acid encoding antigen or prior to such administration, for example, at the time of adoptive transfer or may be administered during the course of the experiment. Administration routes may include subcutaneous, intradermal or intramuscular injection at the same site as administration of nucleic acid encoding antigen or a site distal to the immunisation, topically, orally, intranasally or by infusion from a subcutaneous or intraperitoneal implanted mini pump. For example the additional antigens or immunomodulators or DNA encoding them may be administered using the particle mediated delivery technology.

Uptake of nucleic acid constructs may be enhanced by several known transfection techniques, for example those including the use of transfection agents. Examples of these agents includes cationic agents, for example, calcium phosphate and DEAE-Dextran and lipofectants, for example, lipofectam and transfectam. The dosage of the nucleic acid to be administered can be altered and the T-cell response assessed at the various dosages. Typically the nucleic acid encoding antigen is administered in the range of 1pg to 1mg, preferably to 1pg to 10:g nucleic acid for particle mediated gene delivery and 10:g to 1mg for other routes.

The assessment of the T-cell response can be carried out in a number of different ways. It may be possible to monitor some T-cell responses *in vivo*. Alternatively, T-cells may be extracted from the mammal for example by extraction from a lymph node or the spleen or other lymphoid tissue such as peyers patches and lamina propria in the gut and nasal associated lymphoid tissue. T-cells which have been extracted may be re-stimulated *in vitro* by contact with the antigen.

Subsequently, assessment may be made of a T-cell response such as proliferation of the T-cells or production of cytokines or öther proteins such as cell surface markers by the T-cells or the activity of the T-cells themselves for example by looking at their effect on other dendritic cells, lymphocytes or other cells of the immune system. One or a number of different T-cell responses may be monitored. Clonal expansion of the transferred T-cell population may be monitored. This can be carried out in a number of ways, for example, by separating out lymph nodes from the mammal and using antibodies which target the specific T-cell receptor on the cells to assess the amount of proliferation or clonal expansion of the T-cells.

The T-cells may also be assessed in respect of the proteins such as cytokines that they are producing. Examples of the cytokines which may be monitored include IL-2, IL-5 and interferon-γ. The levels of cytokines and types of cytokines which are being expressed can be monitored using suitable anti-cytokine antibodies. Preferably, T-cells are extracted from the lymph nodes of the mammal following vaccination, and restimulated *in vitro* with the antigen. Cytokine levels in the suspension medium can then be monitored.

Intracellular cytokine levels, cytokine types and ELISPOT analysis of cytokine production may also be carried out. Additional analyses may include assays of cell surface markers on the T-cells such as receptors for cytokines, for example, the IL-2 receptor or markers of T-cell phenotype, for example, to identify memory T-cells. Such analyses may also include use of monoclonal antibodies in ELISA or FACS analysis.

The T-cell responses may be monitored over a period of time with suitable samples being obtained from the mammal at fixed time periods following administration of nucleic acid. The response may be monitored immediately after

administration of nucleic acid. Samples may be taken from 1, 3, 5, 7 days post administration, 2, 3, 4 weeks or 1, 2, 3 months after administration. In some instances the T-cells may be analysed at a much later date such as up to 1-2 years after immunisation. Such analyses may be useful for assessment of memory T-cells. The inventors have surprisingly found that the optimal time after vaccination for measuring peak clonal expansion is delayed for nucleic acid vaccination compared with protein vaccination. The optimal time for measuring clonal expansion is 4-5 days after nucleic acid vaccination compared with 3-4 days after protein vaccination. The inventors further found that the optimal time for measuring cytokines is before clonal expansion, so that the optimal time for measuring cytokines is 3 days, but the optimal time for measuring peak clonal expansion is 4-5 days.

Comparisons can be made between responses generated at each time period.

Preferably, control assays are also carried out to assist in assessing the T-cell responses. For example, a suitable control may comprise administering to the mammal nucleic acid which does not encode the antigen recognised by the T-cell specific receptors. The assay may also be used to assess changes in the T-cell response following changes in the antigen. For example, a small change in the antigen may be shown to affect the T-cell response generated, such as a shift between Th1 or Th2 response.

Preferably, cytokine production, T-cell markers and proliferation of the T-cells is monitored *in vitro* after restimulation of the sample of T-cells with the antigen.

The T-cell responses can be used to determine the effectiveness of a nucleic acid vaccine and also to monitor the type of response which is being generated. For example, clonal expansion of the T-cell population will provide some indication of the effectiveness of the nucleic acid vaccination. In some instances, it may be desirable to perform further analysis on the T-cell population restimulated *in vitro* with the antigen. Such analysis may provide a more accurate assessment of the T-cell response being generated and in some instances can demonstrate responsiveness of the T-cells in the absence of a significant clonal expansion.

The type of cytokine production may provide an indication of the type of T-cell response being generated to a particular antigen. The levels of the cytokines may be used to determine the type of T-cell response and in particular T-cell helper responses being generated. Production of IFN-γ on restimulation *in vitro* is indicative of generation of a Th1 type response. Production of IL-5 is indicative of Th2 type response. The type of T-cell helper response generated may be used to predict whether the vaccine is useful in generating humoral or cellular, such as cytotoxic T-cell responses. The method can therefore be used to monitor whether a particular antigen or nucleic acid is generating a predominantly Th1 or Th2 response. Where it is desired to vaccinate against a viral or other intracellular pathogen, the assay may be used to determine those antigens which generate the most effective cytotoxic T-cell responses. Thus, in a preferred aspect of the invention there is provided a method of monitoring the T-cell helper responses generated following nucleic acid vaccination.

Examples

Construction of plasmids and DNA preparation

The plasmids used are based upon pVAC1, obtained from Michelle Young, GlaxoWellcome, UK, a modification of the mammalian expression vector, pCI, (Promega), where the multiple cloning site, from EcoRI to Bst ZI, has been replaced by the EMCV IRES sequence flanked 5' by unique Nhe I, Rsr II and Xho I and 3' by unique Pac I, Asc I and Not I restriction enzyme sites. The chicken ovalbumin expression plasmid, pVAC1.OVA was constructed by ligating PCR amplified cDNA encoding chicken ovalbumin from pUGOVA, a gift from Dr. F. Carbone, into the expression vector pVAC1.

Plasmid DNA was propagated in E. coli, DH5α, and prepared using plasmid purification kits (QIAGEN Ltd, Crawley, UK), and stored at -20°C at approximately 1 mg plasmid DNA/ml in 10 mM Tris/EDTA buffer.

Cartridge preparation

Preparation of cartridges for the Accell gene transfer device was as previously described (Eisenbraun et al DNA and Cell Biology, 1993 Vol 12 No 9 pp 791-797; Pertner et al). Briefly plasmid DNA was coated onto 2 μm gold

particles (DeGussa Corp., South Plainfield, N.J., USA) and loaded into Tefzel tubing, which was subsequently cut into 1.27 cm lengths to serve as cartridges and stored desiccated at 4°C until use. In a typical vaccination, each cartridge contained 0.5 mg gold coated with between 0.5 – 0.8 μg plasmid DNA.

Mice

Male or female D0.11.10 transgenic mice (6-10 weeks old) were bred in our specific pathogen-free animal breeding facilities at Bury Green Farm. The transgene that these mice express is the T cell receptor (TCR) specific for a chicken ovalbumin peptide residue (residues 323-339; OVA peptide) bound to MHC-II molecule ($I\text{-}A^d$). The monoclonal antibody, KJ1-26 which specifically recognises this TCR is used for identification of TCR-transgenic T cells. Examination of a number of these mice shows that a large proportion (40-65%) of the $CD4^+$ T cells are KJ1-26^+, although a very small population of $CD4^-$ $CD8^+$ KJ1.26^+ T cells are also present (Pape, et al.)

Male or female Balb/c mice (6-8 weeks old) were purchased from Charles River United Kingdom Ltd. (Margate, United Kingdom).

All experiments were carried out under United Kingdom ethical guidelines with mice housed at a constant temperature and humidity, with regular 12 hour cycles of light and darkness, sterile bedding, food and water.

Adoptive transfer

Twenty four hours before immunisation, D0.11.10 splenocytes were adoptively transferred into Balb/c mice at 6-8 weeks of age. Briefly, spleens were collected into ice-cold PBS. The splenocytes were prepared by teasing out into PBS, followed by lysis of red blood cells (1 minute in buffer consisting of 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA), then washing twice in PBS to remove particulate matter, before counting and resuspending as a single cell suspension in an appropriate volume of PBS to give 2.5 x 10^8 cells/ml. Cells were adoptively transferred into the lateral tail vein by injection of 100 μl (ie. 25 x 10^6splenocytes/mouse).

Alternatively, before transfer, splenocytes are treated with anti-CD8 monoclonal antibodies plus complement before transfer to eliminate $CD4^-$, CD8+, KJI-26 + cells that are not ovalbumin responsive and could confuse the analysis.

Immunisations

For DNA immunisations, pVAC1.OVA or pVAC1 (empty vector) was delivered to the shaved target site of abdominal skin by particle-mediated gene delivery (PMGD) from two cartridges using the Accell gene transfer device at 500 lb/in^2 (McCabe WO 95/19799), delivering a total of between 1.0 -1.6 μg DNA per immunisation.

For protein immunisation, mice received a subcutaneous injection at the base of the tail of ovalbumin protein prepared as a suspension 1: 1 in Complete Freunds Adjuvant (Sigma). For each injection a volume of 100 μl was delivered (ie. 100 μg ovalbumin/mouse).

Lymph node collection and cell preparation for ex vivo *assays*

Mice were killed by cervical dislocation from 1-6 days post-immunisation and inguinal and periaortic lymph nodes were collected into ice-cold PBS. Cells were extracted into PBS by squashing nodes between the ground glass ends of sterile glass slides. Cells were separated from debris by removal into new wells. Aliquots (200 μl) were removed for FACS analysis for individual mice. The remaining cell suspensions were pooled for each group of mice and used for *in vitro* T cell proliferation and cytokine analysis.

Analysis of clonal expansion of $CD4^+$ $KJ1\text{-}26^+$ cells

Lymph node splenocytes from each mouse were individually prepared in PBS for analysis. Duplicate samples of each suspension were washed once with staining buffer (PBS containing 0.1% bovine serum albumin and 0.01% azide) in wells of a 96-well plate. One was treated with 50 μl staining buffer containing antiCD4-PE antibody (phycoerythrin-conjugated antiCD4, diluted 1:500; Pharmingen) and FITC (fluorescein isothiocyanate diluted 1:500; Pharmingen) for control. The other sample was treated with 50 μl staining buffer containing

antiCD4-PE (dilution 1:500) and KJ1-26-FITC antibodies (dilution 1:25; Caltag). After 30 minutes at 0-4°C and in the dark, samples were washed 3 times in staining buffer and transferred into tubes for FACS (fluorescence activated cell sorting) analysis (Coulter Epics XL-MCL). The aim of the FACS was to assess the amount of proliferation (clonal expansion) of the $CD4^+$ TCR-transgenic T cells, which had occurred *in vivo* after adoptive transfer and immunisation. A population of cells selected on size, granularity and reflecting index of membrane corresponding to T cells was analysed. $CD4^+$ cells were detected with antiCD4-PE and TCR-transgenic cells were detected with KJ1-26-FITC. The percentage of double-stained cells was measured.

T cell cultures for proliferation and cytokine assays

Suspensions of lymph node splenocytes were pooled for mice from the same groups. Cells were resuspended in medium (RPMI 1640; Life Technology) containing 0.5% syngeneic mouse serum, 2 mM L-glutamine (Life Technology), 100 IU/ml each of Penicillin and Streptomycin (Life Technology), and 50 μM 2-mercaptoethanol, and adjusted to 4×10^6 cells/ml. 100 μl of cell suspension was added to wells of a 96-well plate and re-challenged in triplicate with 100 μl of OVA peptide to give a final dilution range from 1 – 0.0001 μM for proliferation assays, and a single concentration of 1 μM for cytokine assays. Medium without peptide was added to some wells to provide control samples.

For proliferation assays the cells were incubated at 37°C under 5% CO_2 until T cell blasts could be seen in all groups at the highest concentration of peptide (usually 2-3 days), at which time ^3H thymidine (20 μl) was added to all wells and the cells were incubated for a further 16-20 hours. Cells were harvested onto a Filtermat (Wallac) using a semi-automatic cell harvester (Skatron). When dry the filter was placed into plastic bag with scintillant and radioactivity was measured with a liquid scintillation counter (Wallac 1205 Betaplate).

For cytokine assays, cells were incubated as described, with 50 μl aliquots of culture medium supernatants removed at daily intervals from days 1-6 and stored at -80°C for subsequent ELISA analysis.

Cytokine ELISA's

Wells of Maxisorp 96-well plates (Nunc) were coated with 50 µl of anti IL-2, anti IL-5 (both at 0.5 mg/ml) or anti-IFN-γ (1 mg/ml) antibodies and left overnight at 20°C. After washing 3 times with wash buffer (PBS containing 0.05% Tween-20), non-specific binding sites were blocked by a one hour incubation with assay buffer (PBS containing 2% BSA) at 20°C with constant gentle shaking. Following a further 3 washes with wash buffer, 200 µl of supernatant samples (≥1:4 dilution with culture medium) were added and incubated as above for 2 hours before a further 3 washes. Biotinylated antibody solutions (Pharmingen) diluted in assay buffer (1 µg/ml anti IL-2-biotin, 0.5 µg/ml anti-IL5-biotin, or 2 µg/ml anti-IFN-γ-biotin) were added (50 µl/well) and incubated as above for 2 hours. The wells were washed 3 times, HRP-SA (streptavidin coupled with horse radish peroxidase; Caltag) diluted 1:2000 in PBS was added (50 µl/well), incubated as above for 30 minutes, washed a further 3 times, TMB substrate (3, 3', 5, 5' tetramethyl benzidine; Sigma) added (50 µl/well), incubated a further 30 minutes at 20°C while protected from light, then the reaction stopped with 0.25 M sulphuric acid (50 µl/well). Absorbances were read at 450 nm (Molecular Devices 96-well plate reader).

Results

Clonal expansion of the TCR transgenic T cells, measured as the percentage of KJ1-2:6+ CD4+ T cells of the total T cell population in lymph nodes, was detected as early as 3 days after DNA immunisation, reaching peak levels at 4-5 days, after which levels began to decline (Figure 1). The kinetics of clonal expansion following immunisation with protein ovalbumin in CFA was similar, although the percentage of KJ1-2.6+ CD4+ cells reached higher levels compared with DNA immunisation (Figure 1). No clonal expansion was detected for mice immunised with the empty plasmid vector.

The differences in numbers of KJ1-2.6+ CD4+ cells following DNA and protein immunisation was not reflected in proliferation of KJ1-2.6+ CD4+ T cells in response to restimulation *in vitro* with cognate peptide. Indeed, the results suggest higher T cell proliferation for lymph nodes collected from day 4 after DNA immunised, particularly at the lower concentrations of peptide used for restimulation (Figure1 and 2). No proliferation was detected for mice immunised with the empty vector.

IL-2 concentration, measured in culture medium supernatants of lymph node T cells collected from 1- 4 days after immunisation, showed no difference for DNA or protein immunisation (Figure 3). Concentrations increased to peak levels between 2-4 days after *in vitro* restimulation, with the subsequent decline probably due to useage of this cytokine out-weighing production of IL-2 by the cells in culture. Concentrations of IL-2 generated in cultures of lymph node T cells from mice immunised with the empty vector reached levels comparable to DNA and protein immunised groups, the source of which is almost certainly due to stimulation of the basal (ie. non-clonally expanded) level of KJ1-2.6+ CD4+ T cells present from the adoptive transfer without immunisation (Figure 3).

IFN-γ and IL-5 concentrations were measured to provide an indication of T helper 1 (Th1) and Th2 responses, respectively. For IFN-γ, high levels were detected in culture supernatants of lymph node T cells collected 3 and 4 days, with little detected at 5 or 6 days , after immunisation with either DNA or protein (Figure 4). IL-5 was detected in cultures of lymph node T cells collected 3-5 days after DNA immunisation, but only on day 5 and at low levels for protein immunised mice (Figure 5). IFN-γ and IL-5 were not detected in cultures of lymph node T cells collected from mice immunised with the empty vector control.